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An Important Step in *Listeria* Lipoprotein Research▽

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Over the last 10 years, DNA sequences of more than 600 bacterial species have been deposited in databases and are now available to search any gene, motif, or regulatory sequence of interest. Although genome data are instrumental in phylogenetic analysis and in silico design of metabolic and regulatory networks, only a very small fraction of the information has been experimentally validated. A striking example is lipoproteins predicted from genome sequences. Despite the predominance of this class of surface proteins in bacteria (up to 0.5 to 8% of the proteome), very few of these proteins have been identified as lipoproteins by biochemical methods (19). In this issue of the *Journal of Bacteriology*, Baumgärtner et al. (2) report a systematic analysis of lipoproteins of *Listeria monocytogenes*, a facultative gram-positive intracellular bacterial pathogen that causes severe infections (listeriosis) in both human and animals. These authors used an *L. monocytogenes* mutant defective in lipoprotein diacylglyceryl transferase (Lgt), an enzyme involved in lipoprotein processing. Three aspects of their study should be highlighted: (i) new findings concerning the roles of Lgt and lipoprotein-specific signal peptidase II (Lsp) during lipoprotein processing (22); (ii) the identification of 26 of the 68 lipoproteins predicted in the initial annotation of the *L. monocytogenes* strain EGD-e genome (7); and (iii) experimental evidence that a few of these lipoproteins are regulated by PrfA, the master virulence regulator of *L. monocytogenes* (8). Below, we discuss the significance of these findings separately.

Lipoprotein-processing model: differences between gram-positive and gram-negative bacteria. Both gram-positive and gram-negative bacteria contain lipoproteins that are a functionally diverse group of surface proteins. The roles assigned to lipoproteins include substrate binding coupled to ABC transport systems, sensing of environmental signals, antibiotic resistance, respiration, germination, conjugation, adherence to and invasion of eukaryotic cells, control of protein secretion and folding, modulation of the immune response, and maintenance of envelope integrity (20). Lipoproteins are synthesized as precursor forms harboring a signal peptide in the N terminus. Upon processing, lipoproteins are ultimately tethered to the membrane via a lipid moiety, diacylglycerol, which is covalently bound to an N-terminal conserved cysteine residue. Work performed with gram-negative bacteria has indicated that the lipidation reaction, carried out by the enzyme lipoprotein diacylglyceryl transferase (Lgt), is followed by cleavage of the signal peptide (22). The enzyme responsible for the latter reaction is the lipoprotein-specific signal peptidase II (Lsp), which recognizes a genuine L_{-3}-S/A_{-2}-A/G_{-1}-C_{+1} "lipobox." A further modification step, consisting of addition of an N-acyl moiety to the amino group of the N-terminal cysteine, is carried out by the enzyme *N*-acyl-transferase (Lnt). The latter modification takes place after cleavage of the signal peptide by Lsp, which leaves the amino group of the cysteine residue free. Thus, the widely accepted model establishes that enzymes involved in prelipoprotein processing act in a tightly Lgt→Lsp→Lnt order (22). Genome analyses of low-G+C-content gram-positive bacteria, which include *L. monocytogenes*, have shown that an *lnt* gene homolog is not present. Lipoprotein modification in this bacterial group is therefore envisaged as an Lgt→Lsp two-step process (Fig. 1). As Baumgärtner et al. unequivocally demonstrate in their study (2), lack of Lgt activity in *L. monocytogenes* does not preclude cleavage of nonlipidated prelipoproteins by the signal peptidase Lsp. This conclusion was derived from a proteomic analysis showing that in the lipoproteins released into the extracellular medium by the *lgt* mutant the C_{+1} cysteine is the first residue in the N terminus. This protein sequence information demonstrates for the first time that, at least in *L. monocytogenes*, Lsp is able to cleave signal peptide II at the correct position in nonlipidated prelipoproteins. Interestingly, a recent study performed with a *Staphylococcus aureus lgt* mutant revealed that the lipoprotein SitC released into the extracellular medium by this mutant has a molecular weight similar to that of the mature SitC lipoprotein present in membrane fractions of wild-type bacteria (18). This observation indicates that Lsp from other gram-positive bacteria may also be able to cleave the signal peptide in nonlipidated prelipoproteins. Baumgärtner et al. also confirmed that the absence of lipid modification in these proteins does not affect the viability of *L. monocytogenes*, which agrees with previous data reported for *lgt* mutants of *S. aureus*, *Bacillus subtilis*, and *Streptococcus pneumoniae* (11, 12, 18). Lgt is also a dispensable enzyme for *Mycobacterium tuberculosis*, although, as has been shown for *S. pneumoniae*, an Lgt deficiency attenuates virulence (12, 15). Unfortunately, Baumgärtner et al. did not address the role of Lgt in *L. monocytogenes* virulence using animal models, an aspect that certainly deserves study in the future.

Similar to Lgt, the lipoprotein-specific signal peptidase Lsp has been shown to be dispensable for growth in several gram-positive bacteria, including *L. monocytogenes* (6, 13, 21, 23). These findings contrast with those obtained for gram-negative bacteria, in which both Lgt and Lsp are essential enzymes. The presence of two membranes in gram-negative bacteria probably makes accumulation of partially processed prelipoproteins

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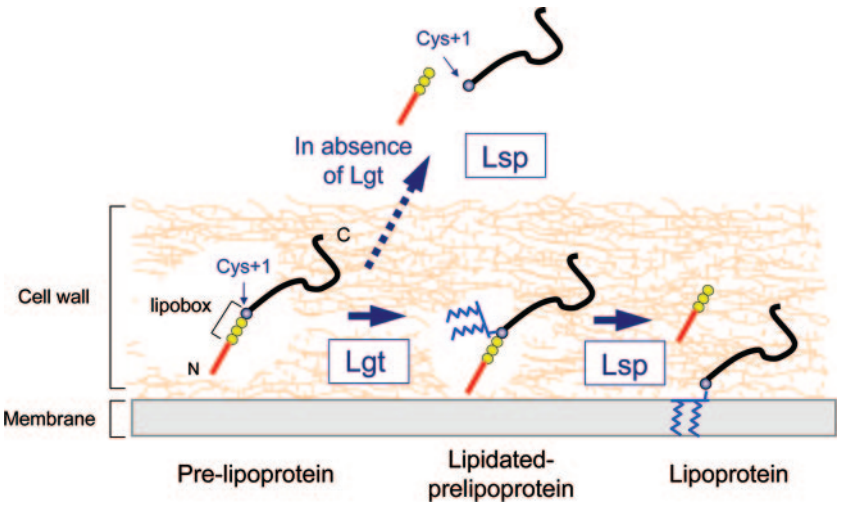

FIG. 1. Lipoprotein-processing model for gram-positive bacteria. The diagram shows the two steps, lipidation and signal peptide cleavage, carried out by the enzymes Lgt, a lipoprotein diacylglyceryl transferase, and Lsp, a lipoprotein-specific signal peptidase II, respectively. As Baumgärtner et al. report (2), an analysis of an *L. monocytogenes* mutant defective in Lgt revealed that Lsp can cleave nonlipidated prelipoproteins at the correct position in the lipobox sequence.

detrimental for the maintenance of basic membrane functions. In the case of Lgt deficiency, gram-positive bacteria might cope with this alteration by releasing nonlipidated lipoproteins into the extracellular medium. Baumgärtner et al. confirmed the contribution of Lsp to the phenomenon by observing that globomycin, an antibiotic that specifically targets this enzyme, prevents such massive release of nonlipidated lipoproteins (2). Release of lipoproteins into the medium was previously reported for *lgt* mutants of *B. subtilis* and *S. aureus* (1, 18). However, in neither of these studies did the authors suspect that Lsp was involved. An intriguing aspect of the study of Baumgärtner et al. extracted from the comparative proteomic analysis is the apparent exclusive release of lipoproteins into the medium by the *L. monocytogenes lgt* mutant. This observation contrasts with what it has been reported for a *B. subtilis lgt* mutant, which, in addition to lipoproteins, releases nonlipoproteins involved in cell wall metabolism (1). The basis of this "unbiased" release of lipoproteins remains elusive, although it was certainly a great help for the systematic identification of lipoproteins.

Massive lipoprotein identification in *L. monocytogenes*. Proteomic analysis of extracellular protein extracts obtained from *L. monocytogenes* wild-type and *lgt* strains resulted in identification of 26 lipoproteins. These lipoproteins included (i) oligopeptide-, amino acid-, and metal cation (iron, manganese)-binding proteins linked to ABC transport systems; (ii) lipoproteins related to pheromone responses involving cell aggregation; (iii) a $CD4^+$ T-cell stimulating antigen; (iv) a putative chaperone-like lipoprotein that assists protein folding; and (iv) lipoproteins having unknown functions. Lipoproteins are the protein class most represented among the predicted surface proteins of *L. monocytogenes* (68 of 133 surface proteins predicted in *L. monocytogenes* strain EGD-e [7]), so the study of Baumgärtner et al. is a major advance in the postgenome functional analysis of this intracellular bacterial pathogen. The sequence information obtained from these 26 lipoproteins allowed the authors to refine previous algorithms used for lipoprotein prediction, such as the algorithm developed in 2002 by Sutcliffe and Harrington using only the 33 lipoproteins validated experimentally that were found during an extensive literature search (19). These numbers provide an idea of the success achieved in the study of *L. monocytogenes* performed by Baumgärtner et al. (2). The authors describe a new hidden Markov model (HMM) as a potent tool for lipoprotein prediction in gram-positive bacteria. The HMM analysis predicted 62 lipoproteins in *L. monocytogenes* strain EGD-e (serotype 1/2 a), and all except one of these proteins (Lmo0810) were included in the initial genome annotation. Interestingly, the study identified Lmo1340 as a new lipoprotein, which was verified experimentally as a protein released into the medium by the *lgt* mutant. The lipobox of Lmo1340 (L_{-3}-F_{-2}-G_{-1}-C_{+1}) is located at the expected distance from the N terminus and differs only slightly from the consensus lipobox, L_{-3}-S/A_{-2}-A/G_{-1}-C_{+1}. The new HMM was also used to predict lipoproteins in two other *Listeria* strains, *L. monocytogenes* F2365 (serotype 4b) and *L. innocua* CLIP 11262. Changes in the previously available information in databases were noted. In the case of strain F2365, the HMM analysis identified 56 lipoproteins, a number lower than the 70 lipoproteins predicted initially. In *L. innocua*, a total of 61 lipoproteins were predicted. A "false-positive" lipoprotein (ortholog of Lmo0810) and a new protein not previously annotated as a lipoprotein in databases, Lin1764, were highlighted. Importantly, the HMM described in the study identified all 33 lipoproteins listed by Sutcliffe and Harrington (19), which makes this algorithm highly attractive for unambiguously predicting lipoproteins in new bacterial genome sequences.

***L. monocytogenes* lipoproteins and virulence.** Two lipoproteins with putative role as solute-binding proteins, OppA and LpeA, were shown to be involved in *L. monocytogenes* virulence. OppA promotes survival in macrophages and in mouse organs, whereas it has been proposed that LpeA mediates bacterial entry into eukaryotic cells (3, 14). Indirect evidence for an important role of lipoproteins in *L. monocytogenes* vir-

ulence was also obtained with an *lsp* mutant, which displayed defects in phagosomal escape upon entry into macrophages (13). This observation suggests that the intracellular infection cycle of *L. monocytogenes*, which includes an early intraphagosomal stage followed by lysis of the phagosomal membrane and intracytosol proliferation, depends on correct insertion into the membrane of an unidentified set of lipoproteins. Many virulence determinants that mediate these intracellular infection stages are regulated by PrfA (8). Baumgärtner et al. analyzed the effect of a *prfA* mutation on the pattern of lipoproteins released by the *lgt* mutant (2). The proteomic analysis revealed smaller amounts of three lipoproteins, Lmo0366, OppA (Lmo2196), and Lm2219, in the extracellular medium of the *lgt prfA* double mutant. Interestingly, a lack of PrfA resulted in two OppA forms having distinct isoelectric points, which led the authors to propose that this lipoprotein may be modified posttranslationally in a PrfA-dependent manner. Another interesting observation was that smaller amounts of Lmo2595, a lipoprotein having no ortholog in the nonpathogenic organism *L. innocua* CLIP 11262, were released by an *lgt prfA* mutant than by the *lgt* single mutant. The authors concluded that this lipoprotein is the first PrfA-repressed protein present specifically in *L. monocytogenes*. However, these data should be interpreted with caution. First, only *L. innocua* strain CLIP 11262 was analyzed. Second, the amount of Lmo2595 in membrane fractions was not determined in the absence and presence of Lgt and/or PrfA. Thus, further experimental evidence is needed to define the putative role of Lmo2595 in virulence. A role as a modulator of the host immune response could be tentatively proposed. Indeed, a recent study performed with an *S. aureus lsp* mutant demonstrated that among the diverse components of the gram-positive cell wall, lipoproteins are dominant compounds with this immunomodulatory role (9).

The in vitro infection assays described by Baumgärtner et al. showed that the *lgt* mutant had a slight defect for proliferation in nonphagocytic cells, which correlated with subtle differences in the growth rate observed in minimal growth medium (2). These data, which parallel those obtained with macrophages and the *lsp* mutant (13), suggest that *L. monocytogenes* might cope with some nutritional stress when it resides in the cytosol of the infected cell. Up-regulation of certain nutrient transporters, such as Hpt, a glucose-6-phosphate translocator, has been shown to mediate rapid proliferation of *L. monocytogenes* inside eukaryotic cells (5). Lipoproteins located on the bacterial surface and involved in acquisition of nutrients might also ensure efficient growth of the pathogen inside eukaryotic cells. This hypothesis clearly deserves further study. Lastly, it is worth mentioning that recent transcriptomic data obtained for intracellular *L. monocytogenes* reflect changes in expression of lipoprotein-encoding genes. Two independent studies reported that lmo0207, which encodes a putative lipoprotein, is upregulated in bacteria located inside host cells (4, 10). Lmo0207 was not identified in the study of Baumgärtner et al., so certain lipoproteins may be produced and function only when *L. monocytogenes* inhabits the host cell cytosol. An exciting future challenge will be to decipher at the protein level the set of lipoproteins synthesized by *L. monocytogenes* during the intracellular infection cycle. Recent successful proteomic studies have provided such relevant information for *Salmonella* and *Chlamydia* proteins synthesized by the pathogens inside eukaryotic cells (16, 17). Undoubtedly, this is a fascinating goal in the postgenome functional era of *Listeria*, which certainly will increase our understanding of how this bacterium has evolved as a successful pathogen.

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